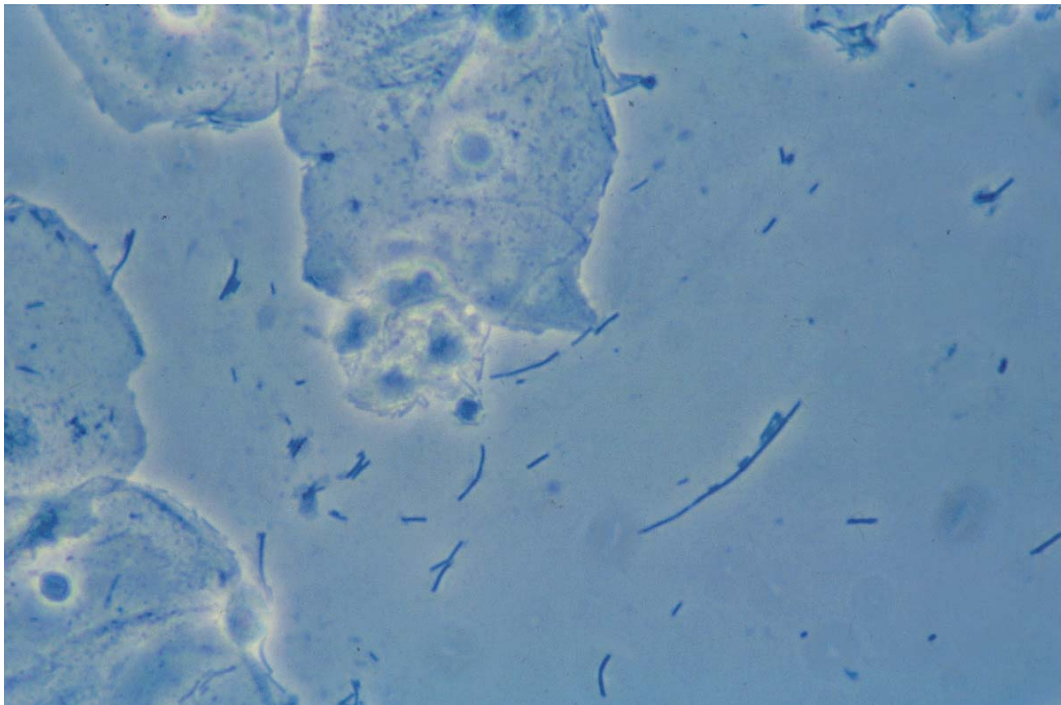


SIMPLE METHOD FOR THE DETECTION OF BACTERIAL VAGINOSIS IN PREGNANT WOMEN

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**Thesis submitted in partial fulfilment of the Master of
Philosophy in International Community Health**

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Dedications

To my father the late Charles Wesley Munjoma.

To two very special people who passed away during the course of this study, my mother Alice Evangelista Munjoma and my uncle the Rev. John Felix “Munatsimirira” Munjoma. May your dear souls rest in everlasting peace.

To my wife Leonissah and children Ruramai, Tapuwa, Tatenda and Taonga.

Abbreviations used

| | |
|---------|--|
| BV | Bacterial Vaginosis |
| SLM | Simple Lactobacillus Method |
| LFGT | Lower Female Genital Tract |
| HIV-1 | Human Immunodeficiency Virus Type1 |
| HSV-2 | Human Simplex Virus Type 2 |
| STI(s) | Sexually transmitted infection(s) |
| OR | Odds Ratio |
| CI | Confidence Interval |
| PPV | Positive Predictive Value |
| NPV | Negative Predictive Value |
| KOH | Potassium Hydroxide |
| pH | Potential of hydrogen ion |
| pap | Papanicolaou |
| RHC | Rural Health Centre |
| DH | District Hospital |
| RPR | Rapid Plasma Reagin |
| TPHA | Treponema Pallidum Haemagglutination Assay |
| ELISA | Enzyme Linked Immunosorbant Assay |
| PCR | Polymerase Chain Reaction |
| GLC | Gas Liquid Chromatography |
| p-value | Probability Value |
| Redox | Reduction-Oxidation |

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Abstract

Objectives: To investigate the use of lactobacillus as a simple tool for the diagnosis of bacterial vaginosis (BV) using wet mounts in pregnant women and to assess the positive predictive value of absence of lactobacillus for Human Immunodeficiency Virus Type-1 (HIV-1) infection.

Methods: 409 pregnant women were enrolled from three randomly selected clinics around Harare. The women underwent clinical examination during which a speculum-aided high vaginal swab was obtained and tested for BV using Amsel criteria, Nugent criteria and the simple lactobacillus method.

Results: The prevalence of BV was 29% by Amsel criteria, 34% by Nugent criteria and 49% by the simple lactobacillus method. The sensitivity and specificity of the simple method using Amsel as the gold standard is 83% and 65% respectively with a kappa value of 0.40. The sensitivity and specificity of the simple test using Nugent as the gold standard is 86% and 82% respectively with a kappa value of 0.68. Sensitivities and specificities of individual Amsel criteria including lactobacillus for determining bacterial vaginosis with Amsel as the gold standard were as follows; discharge 15% and 99%, whiff 96% and 85%, clue cells 96% and 75%, pH 99% and 22% and lactobacillus 83% and 65%. Using Nugent as the gold standard the respective sensitivities and specificities were as follows; discharge 08% and 99%, whiff 70% and 87%, clue cells 67% and 73%, pH 92% 22% and lactobacilli 86% and 82%. The HIV-1 prevalence in the BV study sample (n=392) was about 46%. According to Amsel criteria only 26% (OR= 0.78) of the HIV positive participants have BV while according to the lactobacilli method 59% (OR=2.14) of the HIV positive participants do not have lactobacillus as part of the normal flora of the lower female genital tract. Amsel BV positive predictive value for HIV is 42% while lactobacillus positive predictive value for HIV is 56%.

Conclusion: The sensitivity of the lactobacillus method is as good as Nugent criteria using Amsel as the gold standard. It is much simpler to perform, less expensive, easy to train and takes much shorter time to perform and therefore has a potential for a much wider use than both Amsel and Nugent criteria. The simple lactobacillus method has a better PPV for HIV-1 compared to both Amsel and Nugent. Amsel criteria can be improved by removal of subjective criteria.

Key Words: Lactobacilli, bacterial vaginosis, aerobic vaginitis, Amsel criteria, Nugent criteria, diagnosis, sensitivity, specificity and predictive values.

Chapter I

1. Introduction and Background

1.1 Diagnostic Microbiology

The human body can be described by the types of bacteria which are normally found on or in the different parts of the body. For example the mouth, which is at the beginning of the alimentary canal, has a set of bacteria which is quite different from that found at the other end. There is a variation of bacteria along the length of the alimentary canal. The upper respiratory tract has its own type of bacteria while the lower respiratory tract is supposed to be sterile. The skin, which is the boundary between the inner tissues and the environment, has a typical bacterial population that colonises it. Some of these bacteria play very important homeostatic roles such that their absence may lead to abnormal conditions. Lactobacilli have for a long time been known to be the predominant bacterial flora of the lower female genital tract [1]. They produce lactic acid and hydrogen peroxide which help to keep other bacteria under control [2]. The presence or absence of lactobacilli can therefore be a useful indicator for normal or abnormal conditions in the lower female genital tract. Knowledge of this bacterial distribution, coupled with knowledge of the morphology, pathogenic characteristics, nutritional needs of the bacteria and certain host characteristics form the basis of most microbiological diagnostics. The terms exposure, colonisation, infection and disease are often used to describe the host-microbe relationship and must not be used interchangeably [3].

1.2 Bacterial flora of the lower female genital tract

The female genital tract is divided into two major anatomic regions, the lower female genital tract (LFGT) and the upper genital tract which is normally sterile. The LFGT has an indigenous bacterial flora which can conveniently be divided into two groups, commensal and pathogenic.

The main commensal bacteria are the lactobacilli which are found in concentrations of 10^5 to 10^6 colony-forming units per gram of vaginal fluid while the other commensal bacteria occur in concentrations below 10^5 colony-forming units per gram of vaginal fluid and they account for 10% of the bacterial species recovered from a healthy vagina [4]. These bacteria include *Bacillus spp*, *Corynebacteria spp*, *Diphtheroides* and nondescript Streptococci [5]. In a normal healthy vagina, because of their

relatively bigger size, the area occupied by *Lactobacillus* is far much greater than that covered by the other bacterial species.

The pathogenic bacteria found in the lower female genital tract include facultative aerobes such as *Staphylococcus spp*, *Streptococcus spp* and *Gardnerella vaginalis*, *Escherichia coli* and anaerobes including *Bacteroides spp*, *Prevotella spp*, *Peptostreptococcus spp*, *Mycoplasma hominis* and *Ureaplasma urealyticum* [4, 6]. It is interesting to note that *Mobiluncus spp*, an important bacteria implicated in extreme cases of bacterial vaginosis does not appear neither as a commensal nor pathogen. Perhaps the advent of the Human Immunodeficiency Virus will create more pathogens in the female LFGT.

1.3 Definition of Bacterial Vaginosis

Bacterial Vaginosis (BV) is a disorder of the vaginal microbial ecosystem characterised by a shift in the vaginal flora, from the normally predominant *Lactobacillus spp* to one dominated by a mixed flora including *Gardnerella vaginalis*, *Prevotella spp*, *Porphyromonas spp*, *Bacteroides spp*, *Mobiluncus spp* and genital *Mycoplasma spp*. [7]. This is a limited definition of BV because it does not mention the physiological and biochemical changes that characterise the condition. In view of this, BV can therefore be more comprehensively defined as an alteration in the normal vaginal microbial ecosystem and is characterized by three important features : (a) decreasing numbers of *Lactobacillus* species which produce lactic acid and hydrogen peroxide which in turn inhibits the growth of some pathogenic bacteria, (b) an increase in the concentration of *Gardnerella vaginalis*, curved *Mobiluncus spp*, *Peptostreptococcus spp*, *Mycoplasma hominis* and anaerobic gram-negative rods belonging to the genera *Prevotella*, *Porphyromonas* and *Bacteroides* which produce amines responsible for the fishy odour and (c) a pH greater than 4.5 resulting in the loss of the normal protective acidity of the vagina and subsequent alteration of the normal physiology of the vagina leading to a change in the quality and quantity of the discharge. Between these two definitions there are many variations of the definition of BV but it is important to note that in any definition of BV emphasis is placed on the gradual displacement of hydrogen peroxide-producing *Lactobacillus* species by a variety of other micro organisms, amongst them *Gardnerella vaginalis*. These two organisms are the key players in the making of BV but unfortunately none of them are involved in the diagnosis of BV using Amsel criteria. Although it is a polymicrobial

phenomena one can never have BV without *Gardnerella vaginalis* and with many Lactobacilli! It is therefore quite logical to exploit this knowledge and develop a simple method that can be used for the diagnosis of BV.

1.4 History of Bacterial Vaginosis

Prior to 1955 BV was recognised as a vaginitis whose aetiology was not attributable to a specific agent such as *Trichomonas vaginalis*, *Candida albicans* or atrophic vaginitis, hence the name non-specific vaginitis. Then in 1955 Gardner and Dukes isolated *Haemophilus vaginalis*, an organism which they believed was the causative agent for non-specific vaginitis and subsequently changed the name from non-specific vaginitis to *Haemophilus vaginalis* vaginitis [8]. However, all *Haemophili* spp belong to this genus because basically they require X, V or XV factors for their growth but it was later discovered that the newly named *Haemophilus vaginalis* does not require any of these factors for its growth. Thus the taxonomy of the organism changed from *Haemophilus vaginalis* to *Corynebacterium vaginale*. And so the name of the disease changed from *Haemophilus vaginalis* vaginitis to *Corynebacterium vaginale* vaginitis. That corynebacteria are traditionally gram positive while *Corynebacterium vaginale* is typically gram variable was the reason why the search for a proper name continued. Thus the name was finally changed to its present eponym *Gardnerella vaginalis* [9]. Consequently *Corynebacterium vaginale* vaginitis also changed to *Gardnerella vaginalis* vaginitis.

Three important factors again pushed for a name change. Firstly the suffix *-itis* denotes an inflammation characterised by redness, swelling, pain and the migration of leukocytes to the site of infection. This does not happen in BV and that is perhaps why the majority of women are asymptomatic. Secondly, the pathogenicity of *G. vaginalis* as a sole causative agent of BV was questioned as it could also be recovered from about 50% of women without BV [10]. Thirdly, better culture techniques revealed that anaerobic gram-negative bacilli, gram-positive cocci and genital mycoplasma were also significant microbial components of *Gardnerella vaginalis* vaginitis [6]. Finally the term bacterial vaginosis was adopted at a symposium in 1983. This term recognises that many anaerobic or facultative bacteria are present and that classical signs of inflammation are absent.

A different type of abnormal vaginal flora, aerobic vaginitis, has been recently described [11]. This condition is due to an overgrowth of aerobic bacteria such as

Group B streptococci, *E. coli*, *S. aureus* and others as opposed to an overgrowth of anaerobic bacteria in bacterial vaginosis. The organisms involved in bacterial vaginosis and aerobic vaginitis are indigenous to the LFGT. In both conditions, the dominance of lactobacilli is reduced. Because aerobic vaginitis and bacterial vaginosis are both caused by bacteria one wonders whether the name bacterial vaginosis should be changed to a more specific term, anaerobic vaginosis.

1.5 Signs and Symptoms of BV

Most symptomatic women complain of discharge and an unpleasant fishy-smelling odour which is more noticeable after unprotected sex. The reason why the odour is more noticeable after unprotected sex is because the alkaline semen increases the volatilization of the amines in the same manner that 10% potassium hydroxide (KOH) produces a fishy odour in the whiff test. Odour and discharge are two of the four diagnostic tools used by Amsel in his clinical composite criteria for the diagnosis of BV [12]. But the biggest problem associated with BV is that it is asymptomatic and the discharge is not exclusive to BV unless when it is observed by an experienced clinician! In Zimbabwe Mbizvo et al reported a 30% BV prevalence and that more than 50% of these women did not know that they had BV [13]. The fact that most women are asymptomatic is one of the reasons why this study's objective is to develop a simple inexpensive method for the diagnosis of BV not only in pregnant women but in women of child bearing age who are apparently healthy.

1.6 Risk Factors for Acquiring BV

In Zimbabwe van de Wijgert et al found a strong relationship between intravaginal practices and vaginal flora disturbances, in particular the absence of *lactobacillus* species [14]. Intravaginal practices in Zimbabwe range from finger-cleansing with water to insertion of traditional herbs. Hawes et al site recent douching and having a new sex partner as being associated with acquisition of BV [15]. Other risk factors for BV are level of education, ethnicity, smoking, Intra Uterine Device usage, low socioeconomic status, having a new sex partner, multiple sex partners, increasing parity and frequency of having sex [16]. Sex, however, was never ever intended for anything else except for the single purpose of reproduction. Every other animal except man instinctively observes this fundamental biological phenomenon. That man has sex for pleasure may be the basic cause all STIs that plague mankind all over the world. If sex was purely for reproductive purposes a woman could have sex only

during the fertile period of her menstrual cycle and never during pregnancy and lactation. This may lead to a reduction of the frequency of sex on the part of the woman and a subsequent reduction in the occurrence of STIs including BV which is believed not to be an exclusive STI but somehow caused by sex [17]. BV may be caused by any habit or practice that interferes with the natural ecology of the vagina and to a certain extent unprotected sex may interfere with the natural ecology of the vagina by lowering the vaginal pH. If the frequency of unprotected sex is high then it may mean that the vagina is alkaline for very long periods and this may favour the growth of the bacteria responsible for BV.

There is also evidence of existence of *Lactobacillus*-specific lytic bacteriophages causing the initial microbial shift that characterises BV. In vitro studies have demonstrated that tobacco-associated chemicals, antibiotics and nonoxynol 9 can increase concentrations of these lytic bacteriophages and thereby lead to an increased killing of vaginal *Lactobacilli* [18]. Whatever the risk factor it is important that there be a simple and reproducible method for rapid and inexpensive diagnosis of BV especially in developing countries where the disease is overshadowed by other life-threatening diseases.

1.7 Medical Significance of BV

In a healthy state, the vagina serves as a natural incubator by providing favourable conditions of temperature, moisture, pH and nutrients conducive to the growth of the normal commensal vaginal flora. The vagina serves as a conduit to and from the upper genital tract. As a passage to the sterile upper genital tract and peritoneal cavity the vagina and its indigenous flora play an extremely important role of preventing infections and complications which can conveniently be divided into two groups, gynaecologic and obstetric.

1.7.1 Gynaecologic Complications

BV is related to considerable and possibly preventable infectious morbidity in non-pregnant women. The sequelae of BV now include infertility, endometritis, pelvic inflammatory disease, post-abortion sepsis, post-surgical abortion infections, post-hysterectomy infection, increased risk of HIV and other STIs acquisition.[19, 20]. BV has been suggested as a potential co-factor in the pathogenesis and progression of cervical intraepithelial neoplasia [21] but recent studies have found no association between BV and cervical intraepithelial neoplasia [22]. Bacterial vaginosis is a

potential risk factor for HIV transmission because of the elevated vaginal pH and other biochemical factors thought to impair the host defence mechanisms [23]. As pH increases so does the survival of HIV and thus transmission may be favoured. In Zimbabwe women without lactobacillus had an increased incidence of STIs and were more likely to be HIV positive [13].

1.7.2 Obstetric Complications

BV is a potentially preventable cause of common and costly adverse obstetric outcomes. Many studies from all over the world link BV directly to a number of obstetric complications. These include pregnancy loss, still births, gestational bleeding, preterm birth, preterm labour, premature rupture of membranes, amniotic fluid infection, postpartum endometritis and post caesarean wound infections [5, 24-27]. *Listeria monocytogenes*, a gram positive rod, is also known to cause similar obstetric complications but the difference is that it gains access to the amniotic cavity and membranes through haematogenous spread in the presence of intact membranes [5] rather than by ascension through the vagina.

1.8 BV in Zimbabwe: A Critical Review

Whilst descriptive microbiology of vaginal flora was started by Doderlein in 1894 [1], in Zimbabwe Mason pioneered descriptive work on vaginal flora in 1989 when he described the vaginal flora of women admitted with signs of sepsis following normal delivery, caesarean section or abortion [28]. In that study 20% of women who developed sepsis after delivery had *Gardnerella vaginalis* as indicated by the presence of clue cells. Clue cells, and not BV, were again associated with the development of sepsis in women delivered by caesarean section. In the entire study no direct reference was made to bacterial vaginosis. Instead BV was loosely referred to as *Gardnerella vaginalis* infection and only clue cells were used for the diagnosis. Clue cells are only one of the four clinical criteria developed by Amsel. The Amsel clinical composite criteria was validated in 1983 and this is the method that should have been used for the diagnosis of BV. In 1990 culture of *G. vaginalis* was combined with presence of clue cells for the diagnosis of BV [29]. 63% cultured positive for *G. vaginalis* and had clue cells while 28% cultured positive for *G. vaginalis* and had no clue cells. This shows clearly that growing *G. vaginalis* is a poor indicator for BV. The high prevalence is expected as this was a high risk population.

Between 1995 and 1996 studies investigating intrauterine deaths, stillbirths, intrauterine infections, chorioamniotic infections and neonatal septicaemia were carried out [30-32]. Both studies go all the way to identify the organisms causing the infections but no attempt was made to establish the microbial state of the birth canal of the mothers at some point before or after delivery. Whatever ascending infections that got through to the uterus must have definitely passed through the vagina or the infant must have picked them up during birthing. The vagina serves as a link between the lower genital tract and the sterile upper genital tract. That it is dominated by a specific bacterial population should enable us to develop a simple method that can easily and quickly detect the emergence of abnormal conditions. Such a method would make it very easy to advocate routine screening for BV in pregnant women. There is also need for prospective studies to investigate the effect of BV on the outcome of pregnancy in Zimbabwe more especially after the discovery of a new organism, *Atopobium vaginae*, which was recovered from a tuboovarian abscess[33] and is said to be present in the majority of women with BV[34].

The above studies show either how much BV is overshadowed by the STIs that are secondary to BV infection or the lack of appreciation of the role played by BV in facilitating reproductive tract infections in women, including the deadly HIV virus.

However, current researchers in Zimbabwe seem to have realised the importance of BV and its association with sexually transmitted diseases in particular HIV and its acquisition[13]. Zimbabwe is one of the countries with the highest prevalence and incidence of HIV in the whole world. The HIV prevalence in women with no signs of reproductive tract infections was found to be about 30%[13]. Recent studies also seem to indicate that the prevalence of BV in Zimbabwe is high. In a multi-centred regional study Zimbabwe had the highest prevalence of BV of about 50%[13]. It is therefore not a coincidence that we have the highest BV prevalence and one of the highest HIV prevalence in the world. There must be something in common between these two.

In the study on asymptomatic women where the BV prevalence was about 30% it was disturbing to notice that over 50% of these women did not know that there was something wrong with them [13]. This means that the majority of these women will not seek health care and will therefore continue to be at risk of acquiring and transmitting infections, including HIV-1, each time they have sex. If they become pregnant they are again at risk for the adverse pregnancy outcomes associated with

BV. This problem is compounded by clinicians who are reluctant to probe probably because of the volume of patients that they attend to every day.

There has been no study on the role of BV on adverse pregnancy outcomes in Zimbabwe. A study on neonatal septicaemia in Harare in 1990 did not look for BV as a risk factor and yet those babies that developed septicaemia had a lower birth weight than the control group [32]. BV is not screened for during pregnancy and is not one of the reproductive tract infections that are looked for in the syndromic approach for the diagnosis and management of STIs. This illustrates how much this clinical syndrome is overshadowed by other genital infections such as syphilis and gonorrhoea in Zimbabwe.

The fact that BV is asymptomatic and is associated with many adverse outcomes in sexually active women of reproductive age, including acquisition of HIV, calls for the need for simple screening methods for its diagnosis in developing countries. There is also need to educate Zimbabwean women in general about the physiology of their bodies with the main aim of improving their reproductive health health-seeking behaviour.

1.9 Diagnostic Methods for BV

Various methods are available for the diagnosis of BV. These include culture, wet and dry microscopy, biochemical tests (gas-liquid chromatography) for metabolic by-products of vaginal bacteria, oligonucleotide probe-based hybridization for *G. vaginalis*, multiplex polymerase chain reaction for the identification of BV associated organisms [35], redox potential [36] and rapid or office tests. However the costs and complexity of some methods have restricted them for research purposes and not for routine diagnosis of BV.

1.9.1. Culture.

G. vaginalis culture is a very sensitive method with a very low specificity for the diagnosis of BV and the organism can be recovered from about 36% to 50% of women without clinical signs of BV [35]. In one of the studies in Zimbabwe 91% cultured positive for *G. vaginalis* and 63% had clue cells while 28% had no clue cells [29]. If pH, whiff test and discharge were tested or observed the 63% could further be reduced to 30-40% which is about the prevalence for BV in Zimbabwe. Thus culture leads to over diagnosis and should not be used for directing therapy or as a test of cure after treatment because many women who harbour *G. vaginalis* usually lack any

objective signs of BV. It appears therefore that vaginal cultures for *G. vaginalis* may not be of any use whatsoever in routine diagnosis of BV. However we must always remember that culture played a very important role in the history of BV[8] and may continue to be useful in the identification of new BV-associated organisms such as *Atopobium vaginae*[34] and in research settings.

1.9.2 Wet microscopy.

Wet microscopy provides the simplest, practical, inexpensive and yet objective means for the evaluation of the disturbances of vaginal flora in women. Several techniques are available but the most frequently used are discussed below.

Amsel's criteria, the traditional and reference method for the diagnosis of BV require that any three of the following four criteria be present; homogenous discharge, pH >4.5, fishy odour and clue cells[12]. *Lactobacillus* species, the cornerstone in any definition of BV, are completely ignored in Amsel's composite clinical criteria. The inherent difficulty with Amsel's criteria is that with the exception of pH, the remainder of the criteria are either subjective (discharge and whiff) or potentially technically difficult (clue cells) to judge. Taking Nugent criteria as the gold standard for the diagnosis of BV, the sensitivity and specificity of Amsel's criteria are 70% and 94% respectively [37]. Amsel is a combination of clinical and laboratory observations and that is why it is sometimes referred to as clinical composite. Discharge and pH are observed clinically while clue cells and fishy odour are tested in the laboratory. Because in Zimbabwe clinicians are not trained to look under the microscope one needs at least a nurse and a technician to obtain results and this is not always possible at many health delivery centres in the country. The biggest advantage of this method is that the result can be obtained within ten minutes! If the swab is sent to the lab at the beginning of a gynaecological examination the result will be available by the time the examination is completed. The biggest disadvantage of this method is the need to have a clinician and a technician on site and that the slides do not make a permanent record but this appears to be overcome by the recently introduced rehydrated smear technique [38]. In this technique slides are allowed to dry in the air as if they are intended for gram staining. The following day a drop of normal saline is added to the smear on the slide and a cover slip placed over the smear and examined in the same manner as wet preparations. But work has not been done to establish how long slides can be kept before examination.

In his work on fresh vaginal smears Donders acknowledges the importance of *lactobacillus* in the diagnosis of abnormal vaginal flora and subsequently classified lactobacillary morphotypes into three grades i.e. I, II and III [39]. Grade I contains predominantly *lactobacilli* and represents the normal flora while grade III contains many other bacteria with *lactobacilli* absent. The complexity of this method lies in grade II which he further subdivided into two grades, IIa and IIb. Grade IIa still contains more but reduced numbers of lactobacilli with few small bacteria present while IIb consist of numerous other bacteria with few lactobacilli present. Thus grades IIa and IIb show a shift from *lactobacilli* predominance to the predominance of other bacteria. The two grades may depict what Nugent refers to as the intermediate stage. Schmidt also developed a similar diagnostic criteria in which he weighted small bacterial morphotypes and lactobacillary morphotypes on a 0-8 score scale[40]. A score of 0-1 was regarded as normal, 2-4 as intermediate grade I, 5-6 as intermediate grade II and 7-8 as normal. This method is exactly the same as Donder's except that the Roman numbers in Donders have been replaced by Arabic numbers by Schmidt, as shown in the table 1 below.

Table 1. Comparison of Donders and Schmidt criteria.

| Donders | Schmidt | Interpretation |
|-----------|---------------------------|------------------|
| Grade I | 0-1 | Normal |
| Grade IIa | 2-4 Intermediate grade I | Intermediate |
| Grade IIb | 5-6 Intermediate grade II | Intermediate |
| Grade III | 7-8 | Positive for BV. |

However, the presence of many grades increases the time spent on the microscope and raises the level of expertise required to read the slides. Lack of this expertise would invariably lead to intra and inter reader variability. A modification of the grading system by introducing a cut-off point that determines whether one has normal flora or abnormal flora could make this method more sensitive and easy to use without much technical expertise. The determination of the cut-off point requires careful observations because if the cut-off is placed too low it will exclude some with the disease while if it is placed too high it will include some without the disease. However, there will always be grey zone areas around the cut-off point as some cases struggle to resolve.

1.9.3 Stained smears

The point of departure for the history of BV is the great work by Doderlein where he demonstrated that the normal vaginal flora consists of long gram positive rods which were later named Doderlein's bacteria in his honour [1]. These bacteria we now know today as lactobacilli. Then in 1965 Dunkelberg examined 300 gram stained vaginal smears for "clue cells and dense areas of small gram-negative rods" and reported that all women having clinical signs of BV had gram stain smears consistent with BV[41]. Spiegel recognised the importance of lactobacillus in the diagnosis of BV and subsequently modified Dunkelberg's work in 1983 when his diagnosis was simply made using proportions of *lactobacillus* species, small gram-negative rods, curved gram-variable rods and clue cells[42]. Presence of clue cells indicates full blown BV where most if not all the lactobacillus have been replaced by BV-associated organisms. It is therefore possible to miss diagnosis altogether in those patients who do not have full blown BV. This probably led to the development of Nugent scoring criteria where bacterial vaginosis is put on a 10-point scale where 0-3 is regarded as normal (predominantly *Lactobacillus*), 4-6 intermediate (mixed flora) and 7-10 bacterial vaginosis (no *Lactobacillus*)[43]. The method of arriving at the scores is shown in table 2 below.

Table 2: Scoring chart for Nugent criteria

| Grade | Average number of bacteria per field | score | | |
|-------|--------------------------------------|---------------|---------------|---------------|
| | | Gram positive | Gram negative | Mobiluncus sp |
| 4+ | >30 | 0 | 4 | 2 |
| 3+ | 6-30 | 1 | 3 | 2 |
| 2+ | 1-5 | 2 | 2 | 1 |
| 1+ | <1 | 3 | 1 | 1 |
| 0 | 0 | 4 | 4 | 0 |

Gram positive rods are exclusively lactobacillus while small gram variable and negative bacteria are part of the anaerobic flora responsible for Bacterial Vaginosis.

The final BV score is arrived at by adding the individual points of the three types of bacteria i.e. Long gram positive rods, gram negative short rods and curved gram variable rods. For example complete absence of *Lactobacillus* is grade 0 and scores 4 points while more than 30 gram negative bacilli is grade 4 and also scores 4 points. The Nugent score for this would be $4 + 4 = 8$. *Mobiluncus* species score 1 or 2 points and if they are present the points are added to the 8. Clue cells have no significant role in Nugent criteria which emphasizes the concept of "intermediate" in the diagnosis of BV. These may not have achieved full BV status but the truth is they have shifted

from the normal. They may advance to BV or revert to normalcy but at time of examination it is safer to assume they have BV.

There are difficulties inherent in the Nugent method. Firstly, the interpretation of these smears is subjective because there is always uneven distribution of material on a dry smear and readings may be obtained from different parts of the slide. The microscopic area examined by an oil immersion objective is very small relative to the area covered by the smear. Secondly, over decolourised smears make it difficult to discern the small gram-negative rods. Thirdly, slides are often processed in batches and this may increase the waiting time which most asymptomatic cases are not prepared to do. An added advantage, however, in gram stain-based methods is that the slide provides a permanent record which can be reviewed later.

Because Papanicolaou (Pap) smears are performed routinely it would be of great value if they can be used to screen for BV as well. It has been reported that Pap smears have a sensitivity and specificity of 90% and 97% respectively[44]. However the greatest limitation of pap smears for the diagnosis of BV is that the pap smear sample is a cervical rather than a vaginal specimen. However, it would be quite interesting to note how Nugent criteria would perform when applied to pap smears.

1.9.4 Gas Liquid Chromatography

Gas Liquid Chromatography (GLC) is a measure of metabolic by-products, mainly organic acids, of anaerobic organisms. These organic acids are certainly not produced by *Gardnerella vaginalis*. This technique therefore clearly demonstrated the involvement of other bacteria in the making of BV.

Succinic acid, a metabolic by-product of anaerobic bacteria, is present at higher concentrations among women with bacterial vaginosis[6]. On the other hand, lactic acid produced by lactobacilli, the predominant members of the vaginal flora, is present at high concentrations in women without BV and are colonised by lactobacilli. A succinate/lactate ratio of greater than 0.4, based on gas liquid chromatography (GLC) peaks, was correlated with a clinical diagnosis of BV[6]. This method however is not adaptable for developing countries because of its dependence on expensive and complicated equipment which requires highly trained technicians to operate.

Another GLC-based application is 'the electronic nose,' which detects volatile organic substances in vaginal fluids[45]. The electronic nose diagnostic system is a fully automated system which uses a single swab placed in a sealed envelope. The swabs

are analysed directly without the need for complex extraction procedures. The sample headspace is passed over an array of sensors each of which is specific for different volatile organic compounds based upon size shape and functional group. No matter how fully automated, any GLC system is complex and expensive and therefore not the right technology for the diagnosis of BV especially in developing countries.

1.9.5 Rapid tests

Rapid tests are similar in principle to GLC but have the advantage of not using complicated and expensive equipment. They are based on the detection of metabolic by-products of the micro-organisms that are responsible for BV.

FemExam is a two-card system which detects elevated pH on one spot and trymethylamine on another spot on the same card. A high vaginal swab is touched on both spots and the results are read after one minute. Positives are indicated by colour changes.

Another method, BV Blue test, is a chromogenic diagnostic test for the detection of sialidase enzyme in vaginal fluid. BV Blue detects vaginal fluid sialidase activity at levels of ≥ 7.8 Units where a unit of sialidase activity is defined as the amount of enzyme required to liberate 1 nmol of substrate/ml/min at 37°C[46]. A high vaginal swab is placed in the BV blue testing vessel and incubated for 10 minutes at 37°C after which BV Blue developer solution is added and the colour change is read at once. A blue or green colour indicates elevated levels of sialidase and therefore a positive result while a yellow colour indicates a negative result.

1.9.6 Polymerase chain reaction

A multiplex polymerase chain (PCR) reaction-based diagnostic system has been used to differentiate between bacterial vaginosis due to anaerobic bacteria from any other vaginal disorders [47]. This system is multiplex in the sense that it is detecting many organisms that cause the same condition as opposed to true multiplex PCR systems which detect different conditions at the same time. However the system uses three different primer sets to amplify ribosomal Deoxyribonucleic acid from *Mobiluncus* sp, *B fragilis* group and *G vaginalis* from a high vaginal swab. Detection of the amplified products was done by electrophoresis on 1.8% agarose gel. As in gas liquid chromatography systems, PCR is quite labour intensive and uses expensive and sophisticated equipment which also requires highly specialised personnel. There is absolutely no point in using such methods when simpler and less expensive methods

such as the wet mount based ones are available. Such methods are suitable for neither developing countries nor developed countries and should be used only for research purposes. The other way of making such methods applicable is to be able to detect them concurrently with the detection of other STIs such as *Neisseria* and *Chlamydia*, rather than set up a PCR system for BV only.

1.9.7 Redox Potential

The reduction-oxidation (redox) potential is a physical process that measures the flow of electrons from one terminal to another using a millivoltmeter. Oxygen is the source of the electrons. The redox potential was markedly reduced (up to 200mV) among patients with bacterial vaginosis, demonstrating an oxygen-deficient or anaerobic environment [36]. The redox potential was consistently in the positive range among normal individuals, demonstrating aerobic environment. In my opinion this method however is useful for academic purposes and must not be used for diagnostic purposes.

Table 3 on the next page shows the major advances in the microscopical diagnosis of BV from 1894 to present.

Table 3: Icons in the history and microscopical diagnosis of BV

| Year | Investigator | Diagnostic criteria |
|-------------|---------------------|---|
| 1894 | Doderlein A | Lactobacilli as part of normal vaginal flora (descriptive) |
| 1914 | Curtis A H | <i>Bacteroides sp</i> , <i>Mobiluncus sp</i> and anaerobic cocci as causes of abnormal discharge (descriptive) |
| 1921 | Schroder R | Used gram stains to categorise vaginal flora into normal, intermediate and pathogenic. |
| 1950 | Weaver JD | Confirmed the absence of lactobacilli BV |
| 1955 | Gardner HL Dukes | Isolated <i>Haemophilus vaginalis</i> which they believed caused a new infection which they called <i>H. vaginalis</i> vaginitis. Also described the clinical features of the syndrome. |
| 1965 | Dunkelberg W E | Used gram stains to identify clue cells and dense areas of small gram-negative bacilli. |
| 1977 | Smith R F | Direct microscopy versus culture methods for the identification of <i>H. vaginalis</i> . |
| 1977 | McCormack W M | <i>H. vaginalis</i> was not exclusively associated with abnormal vaginal discharge. |
| 1978 | Pheifer T A | Addition of 10% KOH to release TMA, the amine responsible for the fishy odour. |
| 1980 | Greenwood J R | Name finally changed aponymously to <i>Gardnerella vaginalis</i> |
| 1983 | Spiegel C A | The Spiegel criteria. Gram-negative rods, curved gram-variable rods and fewer than 5 lactobacilli per oil immersion field. |
| 1983 | Amsel R | Amsel criteria. Used clinical features described by Gardner for diagnostic purposes in wet mounts. Discharge, pH, amine odour and clue cells. |
| 1991 | Nugent R P | Nugent criteria. Scoring system for lactobacilli, gram-negative rods and mobiluncus species on gram stains. |
| 1999 | Donders G G | Wet microscopy classification of relative quantities of lactobacilli and other morphotypes into grades I, IIa, IIb and III |
| 2000 | Schmidt H | Morphotypes scoring (1-8) in wet mounts |
| 2002 | Hay P E | Simplified grading of gram stained smears. Introduced two grades, 0 and IV |
| 2004 | Ferris M J | <i>Atopobium vaginae</i> , new organism involved in BV. |

1.10 Some Important Definitions

These are definitions of terms which are often used to describe the performance of screening or diagnostic tests. The contingency four-cell table shown below (table 4) is the basis for calculations of sensitivities, specificities and predictive values.

Table 4: Dummy contingency table for demonstrating calculations.

| | | Disease status | | |
|------|----------|----------------|------------|------------|
| | | Present | Absent | Total |
| Test | Positive | A | B | A+B |
| | Negative | C | D | C+D |
| | Total | A+C | B+D | |

1.10.1 Sensitivity

The sensitivity of a method is the **proportion** (A) of people **with** a disease (A+C) that the method can **correctly** identify as **having** the disease.

$$\text{Sensitivity} = \frac{A}{A+C}$$

1.10.2 Specificity

The specificity of a method is the **proportion** (D) of people **without** a disease (B+D) that the method can **correctly** identify as **not having** the disease.

$$\text{Specificity} = \frac{D}{B+D}$$

1.10.3 Positive predictive value

The positive predictive value (PPV) of a test is the **proportion** of people **with** a disease (true positives) out of all who actually **test positive** by that test. The difference between all those that test positive and true positives is called **false positives** (B).

$$\text{PPV} = \frac{A}{A+B}$$

1.10.4 Negative Predictive value

The negative predictive value (NPV) of a test is the **proportion** of people **without** a disease (true negatives) out of all who actually **test negative** by that test. The difference between all those that test negative and the true negatives is called **false negatives** (C).

$$\text{NPV} = \frac{D}{C+D}$$

1.10.5 Validity

The validity of a method is the degree to which it is capable of measuring what it is intended to measure.

1.10.6 Reliability/repeatability/reproducibility

The reliability of a method is its ability to give consistent results when applied several times under the same conditions.

1.11 Country Profile

Zimbabwe is a Southern African country bordered by Zambia to the North, South Africa to the South, Mozambique to the East and Botswana and Namibia to the West. It has a population of approximately 14 000 000 people. Approximately 3 000 000 people live in Harare and Chitungwiza. It has eight provinces each of which is divided into districts. The districts are further divided into rural zones and each rural zone is further divided into villages. A rural health centre (RHC) is the smallest health delivery facility and is designed to service a rural community within a 10km radius and altogether there are 1500 RHCs in Zimbabwe. The RHC refer patients to a district hospital (DH). There are 57 districts and each district has a DH. Altogether there are 57 DHs. Other districts may have mission hospitals too. The DH and the mission hospital refer patients to the provincial hospitals and there are altogether 8 provincial hospitals. Finally the provincial hospitals refer patients to tertiary hospitals and there are four tertiary hospitals in Zimbabwe.

1.12 Rationale

From the early studies of the characterisation of normal vaginal flora we pick out the importance of Doderlein's bacteria which we now know today as *lactobacillus* [1]. These bacteria are pivotal in any definition of bacterial vaginosis [4, 7]. The importance of *lactobacillus* is taken into consideration in the gram stain-based methods for the detection of BV and hence their improved sensitivity and specificity over Amsel's criteria [12, 35]. This very important organism is completely ignored in the four criteria (elevated pH, discharge, odour and clue cells) that are used by Amsel [12] to diagnose BV in wet preparations. We know that *lactobacillus* species are visible in wet preparations under the microscope at 400X magnification [48]. In this study we wish to exploit the advantages of the gram stain-based [42, 43] and wet preparation methods [12, 48] to come up with a method that is simple, rapid and yet

sensitive for the diagnosis of abnormal vaginal flora and/or BV. Such a method should be very useful for screening purposes in developing countries, especially Zimbabwe, which have a high prevalence of BV [49, 50]. The method should initially be used to screen women into two categories i.e. those with abnormal vaginal flora as indicated by the absence of *lactobacillus* and those with normal vaginal flora as indicated by the abundance of *lactobacillus*. Those women with abnormal vaginal flora, if pregnant, would be considered at high risk for the many adverse pregnancy outcomes associated with BV. Identification and subsequent treatment of BV in women may reduce STIs including HIV which has caused untold suffering to many families in Zimbabwe.

The purpose of this study therefore is to develop a simple method with high sensitivity and specificity which can be used for the identification of women with abnormal vaginal flora or BV and without much technical expertise. This method will be used not only in Zimbabwe but in all developing countries.

1.13 Objectives

1.13.1 General Objective

To develop a simple, rapid and versatile method with a high sensitivity and specificity for the detection of abnormal vaginal flora and/or BV for use at peripheral health delivery centres in Zimbabwe as well as other developing countries.

1.13.2 Specific Objectives

1. To investigate the use of *lactobacillus* as a simple tool for the diagnosis of bacterial vaginosis in pregnant women using fresh vaginal samples.
- 2 To assess the impact of *lactobacillus* as a criterion on the sensitivity and specificity of Amsel diagnostic criteria.
3. To compare the prevalence of HIV-1 between pregnant women who have *lactobacillus* and those who do not have *lactobacillus*, and between those who have BV and those who do not have BV in order to predict the risk of HIV-1 infection.

Chapter II

2. Methodology

2.1 Study design

This is a cross-sectional laboratory-based study where fresh vaginal samples were collected from pregnant women for the assessment of lactobacillus as a diagnostic tool for BV in Zimbabwe. The BV status of the women was determined by Amsel's criteria which is regarded as the gold standard for the diagnosis of BV [12]. Samples were collected from the pregnant women during the third trimester. The sensitivity of lactobacillus alone and in combination with pH, and clue cells was also compared with the gram stain-based Nugent's criteria. The study was part of an ongoing study where the motor development of infants born to HIV positive mothers on Nevirapine is being investigated with infants born to HIV negative mothers as controls.

2.2 Study area

The study was conducted at three randomly selected clinics near Harare in Zimbabwe. The clinics are Epworth polyclinic in Epworth, St Mary's polyclinic and Seke North clinic both in Chitungwiza.

2.2.1 Epworth Clinic

Epworth is a poor peri-urban settlement situated about 10Km due east of Harare. It is administrated by the Epworth Local Board. The population of Epworth is approximately 87 625 according to the 2002 census. Most of the people in Epworth work in Harare. Some of the people are self-employed. This settlement was established by a religious organisation in the early seventies as many people fled from their rural homes. Epworth polyclinic is the only health centre that has maternity facilities in Epworth. It handles about 3000 deliveries per year at an average of about 250 per month. Complicated cases are referred to Harare Central Hospital.

2.2.2 St Mary's Clinic

St Mary's is a low income high density residential area in the town of Chitungwiza, which is situated about 20km south east of Harare. It is administered by Chitungwiza Town Council. The population of St Mary's is approximately 84 587 according to the

2002 census. The clinic is the only health centre that handles deliveries. St Mary's clinic handles about 1500 deliveries per year. Complicated cases are referred to Chitungwiza General Hospital.

2.2.3 Seke North clinic

Seke north shares the same catchments area with St Mary's clinic. It is under the same Chitungwiza Town Council and also refers patients to Chitungwiza General Hospital.

2.3 Study Population

The study population is composed of pregnant women in their third trimester that had already been enrolled in the ongoing Nevirapine study. Age was not a restriction for enrolment as long as they were pregnant. The women were permanent residents of either Epworth or Chitungwiza and were registered to deliver at any of the three study clinics.

2.3.1 Study unit

The study unit for this study was the pregnant woman.

2.4 Selection criteria

As part of an ongoing main study, the BV study used participants which had been enrolled using inclusion and exclusion criteria which are listed below.

2.4.1 Inclusion Criteria

- pregnant (3rd trimester) and attending ANC clinic
- living in either Epworth or Chitungwiza
- known HIV-1 serological status
- intending to deliver at either Epworth, St Mary's or Seke North Polyclinics

2.4.2 Exclusion Criteria

- used any antibiotics in the last two weeks.
- used vaginal creams in the last two weeks.
- ever douched or practiced intravaginal techniques such as finger cleansing.
- had sexual intercourse within the last 8hrs
- had a history of previous pregnancy complications

2.5 Sample size calculation

The minimum number of participants required for this study was calculated using the formula $n = z^2pq/d^2$ where;

n = minimum number required.

z = the level of statistical significance of the expected result, in this case 1.96 for 95% confidence interval.

p = the prevalence of the disease or condition. In Zimbabwe the prevalence of BV is about 30% [13].

q = 1-p

d = maximum allowable error which is normally put at 0.05.

$$\begin{aligned}\text{Therefore } n &= \frac{(1.96)^2 \times 0.3(1-0.3)}{(0.05)^2} \\ &= \frac{3.8416 \times 0.21}{0.0025} \\ &= \underline{\underline{323 \text{ participants}}}\end{aligned}$$

2.6 Sampling Method

Three clinics were randomly selected from a total of seven clinics in and around Harare and Chitungwiza. Participants were enrolled sequentially from January 2003 to December 2003 if they met the inclusion criteria.

2.7 Subjects

409 participants were enrolled from the three clinics as follows, 135(33%) from Epworth, 141(34%) from Seke North and 133(33%) from St Mary's clinic. Out of the 409 participants 17(4.2%) were missing Amsel data and were therefore removed from the sample leaving 392 participants whose distribution in the clinics were as follows; Epworth 132(34%), Seke North 132(34%) and St Mary's clinic 128(32%). The participants were all enrolled during the third trimester. The range of the duration of their pregnancies was between 28 weeks and 40 weeks. The participants had been pregnant (gravida) between 1 and 5 times while they had given live births (para) up to 5 times.

2 participants had their ages missing and were therefore excluded from all the age calculations. Thus only 390 participants were available for age calculations.

2.8 Data collection

Three types of data were collected. These are demographic, laboratory and clinical data.

2.8.1 Demographic data

A questionnaire was developed to collect demographic data but responses to this questionnaire were extracted from the parent study questionnaire because we did not want to subject the participant to another interview as this would impact negatively on subsequent follow up visits of the parent study. This demographic data was used primarily to describe the sample population.

2.8.2 Laboratory data

Three sets of laboratory data were collected. The first set of data was for the diagnosis of BV using Amsel criteria and the second set was for the diagnosis of BV using Nugent criteria. The final set of data was for the new simple lactobacillus method.

2.8.2.1 Amsel criteria

A high vaginal swab was collected during the pelvic examination and it was used to perform the whiff test and microscopic examination of clue cells in the laboratory [12]. Amsel criteria need an independent assessment of four individual criteria and a positive Amsel test requires the presence of any three of the four criteria which are pH, whiff, discharge and clue cells.

2.8.2.2 Nugent criteria

Bacterial morphology was evaluated from a gram stained vaginal smear using the 100X objective (oil immersion) of a compound light microscope. Three types of bacterial morphologies are used for this evaluation. They are thick gram positive rods indicative of lactobacilli species, short slender gram variable rods (*Bacteroides*, *Prevotella* or *Gardnerella* spp) and curved gram variable rods characteristic of *Mobiluncus* species. These bacteria are referred to as the Nugent bacteria in this document. A score of 0-10 was assigned in light of the relative proportions of the above mentioned bacterial groups. A score of 0-3 was normal while a score of 7-10 was positive for BV and a score of 4-6 was considered to be intermediate [43].

2.8.2.3 The Simple Lactobacillus method.

The presence or absence of lactobacilli was noted in fresh high vaginal samples using the 40X objective and 10X eye pieces of a compound light microscope. All women

with BV have few or no lactobacilli in the LFGT. The absence of lactobacilli can therefore be used to indicate the presence or absence of BV.

2.8.2.4 Laboratory tests for Sexually Transmitted Infections.

Trichomonas vaginalis was diagnosed by observing characteristic movements in a wet smear using a compound light microscope at a magnification of 400X. Yeast buds and pseudohyphae were also examined in a wet smear to which a drop of 10% KOH was added. Syphilis was screened first with RPR (rapid plasma reagin) and the positive ones were confirmed with TPHA (Treponema Pallidum Haemagglutination Assay). Herpes Simplex Virus type 2 (HSV-2) was tested using an Immunoglobulin G (IgG) based enzyme linked immunosorbent assay (ELISA). Human Immunodeficiency Virus type 1 (HIV-1) was screened using a rapid test called Determine manufactured by Abbott diagnostics and confirmed by another rapid test, Capillus, manufactured by Cambridge Biotek. RPR, TPHA, HSV-2 ELISA and HIV-1 are all serological tests.

2.8.3 Clinical data

Vaginal discharge and pH are two of the four criteria required for the diagnosis of BV using Amsel's criteria which is sometimes referred to as the clinical composite criteria. These were collected during a pelvic examination.

2.8.3.1 Vaginal discharge

Discharge was observed before and after inserting a non lubricated sterile speculum into the vagina. Discharge was reported as positive if it was thin, adherent, homogenous and milky whitish. Other types of discharges, if present, were also noted.

2.8.3.2 Hydrogen ion potential (pH)

The lateral wall of the vagina nearer the cervix was touched with a gloved hand which had a spot for reading pH. The colour change was matched with a colour coded guide provided by the manufacturer.

2.9 Sample collection and transport

A sterile non-lubricated speculum was gently inserted into the vagina during a pelvic examination by a qualified clinician. Two sterile cotton-tipped swabs were labelled with the participant's main study identification number and used to obtain two samples of vaginal fluid both from the posterior fornix. One drop of normal saline was put on one swab soon after collection to prevent the swab from drying in the heat. The

swab was recapped and all swabs to which normal saline was added were sent to the laboratory around 14:00hrs the same day. These swabs were used for the diagnosis of BV using Amsel criteria and the Simple Lactobacilli method.

The second swab was used to prepare a vaginal smear on a microscope slide. The microscope slide was air-dried and stored in slide boxes. These slides were later sent to the laboratory at the end of the week for gram staining and subsequent diagnosis of BV using Nugent criteria.

2.10 Sample preparation

2.10.1 Wet preparations (Amsel Criteria and Lactobacillus method)

Two drops of normal saline (approximately 200uL) were placed on either sides of a glass slide using a 3ml transfer pipette. The drops were placed about 1cm from the ends of the slide so that they must not flow into each other at any stage during the process.

The swab was removed from its sheath and rehydrated with two drops of normal saline. The swab was mixed gently in a circular motion several times in the drop on the left side of the glass slide. After mixing the swab was lifted and rotated through 180 degrees and again mixed gently in a circular motion several times in the drop on the right side of the glass slide. The rotation through 180 degrees was to ensure that both drops received similar amounts of vaginal substances. The used swab was put back in its sheath and kept until the examination was complete when it was discarded in a biohazard container. A cover slip was gently placed on the drop on the left side of the glass slide. This drop was called the saline preparation. A drop of 10% potassium hydroxide (KOH) was added to the drop on the right side of the glass slide and at once whiffed for the liberation of a fishy odour indicative of the presence of volatile amines such as trimethylamine. Another cover slip was also placed on this drop and it was referred to as the KOH preparation. The whiff result was recorded as either positive or negative and recorded on the Amsel record sheet before the slide was examined under the microscope. The saline preparation was then put under the microscope and viewed for the presence or absence of clue cells under the 40X objective. The reason for recording the whiff test before examining the slide for clue cells was to make the two results independent of each other although done by the same person.

The same saline preparation was also examined for the presence or absence of lactobacillus species and *T. vaginalis* [39] using the 40X objective.

2.10.2 Dry smears (Nugent criteria)

One of the two high vaginal swabs was used to prepare a dry vaginal smear by rolling it along the middle portion of the slide. The smear was air-dried and delivered to the laboratory for gram staining in slide boxes.

The slides were processed in batches of eight to ten. They were put back to back in a staining trough and fixed in 100% methanol for two minutes. The slides were removed from the staining trough, air-dried and then placed on a staining rack with the smear side facing up and the entire slide flooded with crystal violet, the primary stain, for one minute. The slides were washed one by one in gentle running tap water. The slides were again placed face up on the staining rack and flooded with lugol's gram iodine solution for another minute. They were washed one by one with gentle running tap water and decolourised by adding acetone drop by drop until all the blue colour (crystal violet) had run out. The slides were washed once more in tap water and counterstained with safranin for 30 seconds. They were rinsed for the last time and blotted with absorbent paper and left to completely dry in the air.

The slides were examined using the immersion oil objective and scored for BV using Nugent criteria. The slide was first scanned to get an impression of what bacteria were present and then the numbers of the Nugent bacteria in five representative fields only were counted and then averaged in order to obtain points for each type of bacteria. For example between 6 and 30 lactobacilli gets 1 point while the same number of small gram variable rods get 3 points. All the points were then added to obtain the BV score which can be negative (0-3), intermediate (4-6) and positive (7-10). The example given above would get a score of 4 and would therefore fall in the intermediate category. See section 1.9.3 (table 2) for detailed description of the scoring system.

2.11 Internal control

During staining, *Escherichia coli* and *Staphylococcus epidermidis* which are known gram-negative and gram-positive organisms respectively were included to control the procedure. The staining procedure was valid only when these organisms were their true gram reaction.

All the slides were then examined by two technicians (M and G). Results were either discordant or concordant. Concordant results were taken to be the BV result for that participant. Discordant results were independently examined by a third technician (O). A similar result obtained by any two technicians became the final BV result. Slides

with three different results were called discrepant and thus not included in the final analysis, as slides with an intermediate result.

2.12 Statistics

Data was transcribed from the primary data collection sheets into an excel program. The excel data was later imported into Statistical Package for Social Scientists (SPSS) version 11.0 which was used to calculate frequencies and prevalence. Chi-square test was used to test association significance and odds ratios (OR) between HIV and BV groups. 95% confidence intervals on selected proportions, sensitivities, specificities and predictive values were calculated manually from SPSS-generated contingency tables.

2.13 Ethics

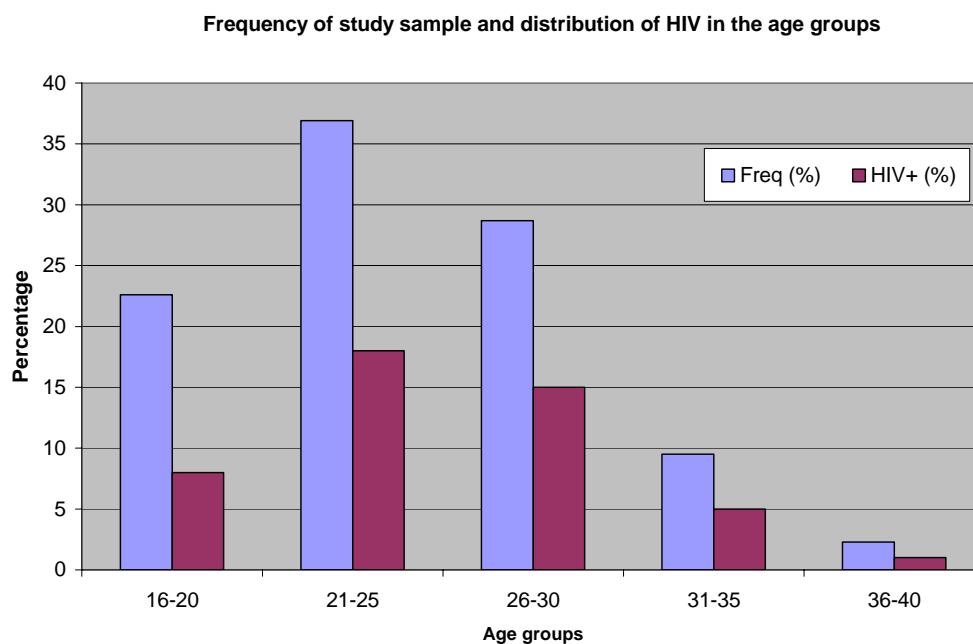
Both the Medical Research Council of Zimbabwe and the Norwegian Ethical Committee approved the study. Participants were issued with identity numbers to mask their true identity. These identity numbers were used on all specimens collected, including the high vaginal swab for this study. The consent forms bearing client's names and addresses together with study records were locked away and only available to study personnel. The participants were informed that participation in the BV study was of their own free will and that they were free to exit the BV study at any time without either jeopardising their participation in the parent study or their treatment at the clinic.

Chapter III

3. Results

Data for analyses were available for 390 participants whose ages ranged between 16 and 40 years with a mean age of 24.57 years and a standard deviation of 4.88 years. 182(46%) of the BV study participants are HIV positive while 210 are HIV negative. The participants were stratified in 5-yr age groups and the prevalence of HIV-1 in the age groups is shown in fig 1 below.

Figure 1. Frequency of the 390 participants in age groups (blue/light) and prevalence of HIV (purple/dark) in the same age groups.



45 (11.5%) participants were intermediate according to Nugent criteria. Three different Nugent results were obtained on 29 participants and therefore a common result could not be reached. All the 74 (19.1%) participants were excluded from all analyses involving Nugent criteria. Thus the total number of participants for Nugent analyses is 318 while for Amsel is 392.

Amsel, Nugent and the Simple Lactobacilli Method.

Table 5: Prevalence and 95% confidence interval of bacterial vaginosis by the three methods.

| Method | Prevalence | 95% Confidence Interval |
|----------------------|------------|-------------------------|
| Amsel | 29 | 25-33 |
| Nugent | 34 | 29-39 |
| Simple Lactobacillus | 49 | 44-53 |

The prevalence of BV using the three different methods is given above in table 5 above. According to Nugent 45 participants were in the intermediate category while 30 participants were discrepant. Of the 45 Nugent intermediate participants 16 were classified as BV positive and 29 BV negative by Amsel criteria while 25 were classified as BV positive and 20 BV negative by the SLM. The level of agreement in the intermediate group between the SLM and Amsel criteria is kappa 0.01.

The sensitivity and specificity of the SLM compared to Amsel criteria as the gold standard is 83% (95% CI 79%-87%) and 65% (95% CI 60%-70%) respectively with a kappa index of 0.39. The positive predictive value (PPV) and negative predictive values (NPV) are about 49% (95% CI 44%-54%) and 91% (95% CI 88%-94%) respectively. Table 6 was used to calculate sensitivities, specificities and predictive values.

Table 6: Cross tabulation between Amsel criteria and the Simple Lactobacilli method.

| | | Amsel Criteria | | Total |
|--------------|----------|-----------------------|-----------|--------------|
| | | Positive | Negative | |
| SLM | Positive | 93 | 98 | 191 |
| | Negative | 19 | 182 | 201 |
| Total | | 112 | 280 | 392 |

SLM, Simple Lactobacilli Method.

The sensitivity and specificity of Nugent versus Amsel as the gold standard is 82% (CI 78%-86%) and 72% (CI 67%-77%) respectively with a kappa index of 0.46. The PPV and NPV are 53% (CI 48%-58%) and 91% (CI 88%-94%) respectively. The intermediate group and discrepant slides are excluded. Calculations based on table 7.

Table 7: Cross tabulation table between Amsel criteria and Nugent criteria.

| | | Amsel Criteria | | Total |
|------------------------|----------|-----------------------|----------|--------------|
| | | Positive | Negative | |
| Nugent Criteria | Positive | 71 | 64 | 135 |
| | Negative | 16 | 167 | 183 |
| Total | | 87 | 231 | 318 |

The sensitivity and specificity of the Simple method against Nugent criteria as the gold standard is about 86% (CI 82%-90%) and 82% (CI 78%-86%) respectively while the PPV and NPV are 78% (CI 73%-83%) and 89% (CI 86%-92%). The level of

agreement is Kappa 0.68. Again the intermediate group and discrepant slides are excluded. Calculations based on table 8.

Table 8: Cross tabulation table between Nugent criteria and the Simple Lactobacilli method.

| | | Nugent criteria | | Total |
|--------------|----------|------------------------|----------|--------------|
| | | Positive | Negative | |
| SLM | Positive | 116 | 32 | 148 |
| | Negative | 19 | 151 | 170 |
| Total | | 135 | 183 | 318 |

SLM, Simple Lactobacilli Method.

Performance characteristics of individual criteria.

The sensitivities, specificities and predictive values of lactobacilli and other individual Amsel criteria i.e. discharge, whiff test (amine test), clue cells and pH when compared with Nugent criteria are shown in table 9 below.

Table 9: Performance characteristics of individual Amsel criteria and lactobacilli for the diagnosis of bacterial vaginosis using Nugent criteria as the reference method.

| Criterion | Positive(%) | Negative | Sens. | Spec. | PPV | NPV |
|------------------|--------------------|-----------------|--------------|--------------|------------|------------|
| Discharge | 13 (4) | 295 | 08 | 99 | 85 | 58 |
| Whiff | 118 (37) | 199 | 70 | 87 | 80 | 80 |
| Clue cells | 140 (44) | 177 | 67 | 73 | 65 | 75 |
| pH | 256 (81) | 49 | 92 | 22 | 47 | 76 |
| Lactobacilli | 148 (47) | 169 | 86 | 82 | 78 | 89 |

Sens, Sensitivity; Spec, Specificity; PPV, Positive predictive value; NPV, Negative predictive value.

Any three of discharge, whiff, clue cells and pH ≥ 4.5 represent the conventional Amsel criteria. The prevalence of bacterial vaginosis using this method is 28.6%. When discharge is replaced by lactobacilli, the prevalence of BV is 39.8% while replacing whiff by lactobacilli the prevalence is 31.9%. A participant is positive for BV when any three out of the four criteria are positive, as in the original Amsel criteria. The sensitivities, specificities and predictive values of these three combinations with Nugent as the reference method are shown in table 10 below.

Table 10: Performance characteristics of the three combinations using Nugent as the reference method.

| Criteria (any 3 out of 4 is positive) | Sensitivity | Specificity | PPV | NPV |
|--|--------------------|--------------------|------------|------------|
| Discharge, Whiff, Clue cells, pH | 53 | 91 | 82 | 72 |
| Whiff, Clue cells, pH, lactobacilli | 76 | 88 | 82 | 83 |
| Discharge, Clue cells, pH, lactobacilli | 61 | 91 | 84 | 72 |

PPV, Positive predictive value; NPV, Negative predictive value.

HIV, BV and Lactobacilli.

182(46%) of the BV study participants are HIV positive while 210 are HIV negative. The HIV negative participants served as the comparison group. According to Amsel criteria only 47 (26%), 95% CI of 20-32, p-value = 0.313, Odds Ratio (OR) = 0.78 of the HIV positive participants have BV while according to the Simple Lactobacilli Method 107 (59%), 95% CI of 52-66, p-value < 0.0001 OR = 2.14 of the HIV positive participants do not have lactobacillus as part of the normal flora of the lower female genital tract.

The figures for Nugent criteria exclude 74 participants, i.e. 45 intermediate and 29 with discrepant results. Thus there were 145 participants who were HIV-1 positive and 173 participants who were HIV-1 negative, making a total of 318 participants. 76 (52%), 95% CI of 44-60, p-value 0.001, OR 2.13 of the HIV-1 positive participants have BV.

The positive predictive values of Amsel BV, Nugent BV, Simple Lactobacilli BV and HSV-2 are as shown in table 11. Absence of lactobacilli is indicative of disturbance of vaginal flora or bacterial vaginosis.

Table 11: Proportions of Amsel BV, Nugent BV, Simple Method BV and HSV-2 in the HIV-1 positive and negative groups and their predictive values for HIV-1.

| | <u>HIV-1 Status</u> | | Total | PPV |
|------------------------|----------------------------|-----------------|--------------|------------|
| | Positive(%) | Negative | | |
| Amsel BV | 47(26) | 65 | 112 | 42 |
| Nugent BV | 76(52) | 59 | 135 | 44 |
| Simple Lactobacilli BV | 107(59) | 84 | 191 | 56 |
| HSV-2 | 143(76) | 75 | 218 | 66 |

HIV-1, Human Immunodeficiency Virus Type 1; HSV-2, Herpes Simplex Virus Type 2; BV, Bacterial Vaginosis; PPV, Positive Predictive value; NPV, Negative Predictive value.

HSV-2 results were available for 370 participants. 218 of the 370 participants were HSV-2 positive, therefore giving an HSV-2 prevalence of 59%. Of the 218 that were HSV-2 positive 63(29%) had BV (OR = 1.22) according to Amsel criteria while 115(53%) had no lactobacilli (OR = 1.54) as part of their vaginal flora.

The prevalence of BV according to age among the HIV positive participants and the HIV positive predictive values using different diagnostic methods are given in table 12 below.

Table 12: BV prevalence and HIV positive predictive values using different BV diagnostic methods.

| Age group | <u>HIV+</u> | <u>BV Lacto</u> | | <u>BV Amsel</u> | | <u>Nugent</u> | <u>BV</u> |
|-----------|-------------|-----------------|-----|-----------------|-----|---------------|-----------|
| | Freq | Pos | PPV | Pos | PPV | Pos | PPV |
| 16-20yrs | 32 | 22(69) | 46 | 07(22) | 25 | 17(66) | 46 |
| 21-25yrs | 70 | 42(60) | 61 | 21(30) | 50 | 33(63) | 62 |
| 26-30yrs | 57 | 29(51) | 60 | 02(21) | 44 | 17(30) | 62 |
| 31-35yrs | 19 | 12(63) | 63 | 06(32) | 60 | 08(61) | 66 |
| 36-40yrs | 04 | 02(50) | 33 | 01(25) | 25 | 01(61) | 20 |
| Total | 182 | 107(59) | 75 | 47(26) | | 76(52) | |

Pos, positive; PPV, Positive Predictive Value. NB. The prevalence and PPV for Nugent BV are calculated using different figures because of the exclusion of intermediate and discrepant slides. The number of HIV-1 positives in the respective age groups, starting with the 16-20 yr age group is 26, 52, 51, 13 and 3. The total number of HIV-1 positives = 145. See how these compare with figures under HIV+ in the table.

3.1 Discussion

Amsel, Nugent and the Simple Lactobacilli Method.

Both the Amsel and Nugent criteria were used as reference methods for the evaluation of the Simple Lactobacillus Method (SLM) although Amsel is regarded as the “gold” standard [51]. Both methods were used because of the need to highlight the differences in performance of these methods when different sets of conditions apply. Since Amsel and Nugent are frequently used as reference methods we feel that these differences in performance under different conditions may affect the sensitivity, specificity and predictive values of methods under evaluation.

In his original article Amsel defines a pH > 4.5 as one of the four criteria for the diagnosis of bacterial vaginosis [12]. This clearly excludes pH values that are equal to 4.5. However, others have used pH values ≥ 4.5 [52] while others have used a pH ≥ 4.7 [15, 53]. Some have ventured to use pH values ≥ 5.0 as a cut-off for Amsel criteria [54]. These variations in pH cut-off values have an effect on the overall performance of the test and also on the prevalence of the disease being investigated. When the sensitivity and specificity of a diagnostic method is altered it follows that the prevalence and incident measurements of the disease are altered and subsequently any methods evaluated against it are also affected. In this study when pH was changed by only 0.1 pH unit, from ≥ 4.5 to a pH > 4.5, the sensitivity, specificity and predictive values of the SLM changed from 83%, 65%, 49% and 91% to 86%, 61%, 38% and 94% respectively. The prevalence of bacterial vaginosis as measured by Amsel changed from 28% to 21%. Depending on the type of disease and magnitude of the change this may cause public health officials to make inappropriate decisions which

may lead to regrettable consequences. It therefore appears that even these very objective criteria cited by Amsel can be made to perform subjectively, thereby further eroding the very basic properties of Amsel criteria that have established it as a reference method in the diagnosis of BV.

Looking closely at table 6 in the result section we notice that the number of false positives (98) outnumber the true positives (93) by more than 100%. This tells us that our method is picking up not only BV but other conditions that are not picked up Amsel criteria. We know that absence of lactobacilli is not synonymous with BV but we also know that absence of lactobacilli is a positive indicator of vaginal flora disturbance. Aerobic vaginitis is one form of vaginal flora disturbance that is signalled by replacement of lactobacilli by aerobic organisms such as Group B streptococci, *E. coli* and *S. aureus* [11] and it may be possible that the SLM is also picking up this condition. The false positives also include some of the participants with only one or two criteria and therefore fail to count as positives using Amsel criteria, i.e. any three out of four. The overall effect of the false positives outnumbering the true positives is to reduce the PPV of the SLM which in this case is barely 49%. This PPV is only for BV and not for vaginal flora disturbance.

When the SLM is compared with the Nugent criteria the sensitivity, specificity, positive and negative predictive values are 86%, 82%, 78% and 89% respectively. The Kappa index is 0.68. The figures look better but this is because the intermediate group was not included in the analysis. The Nugent intermediate group is difficult to diagnose and is the source of many inter-reader variations [55]. Different scoring combinations in the intermediate group may lead to the same points. Since the slides are judged by the total points and not by the individual scores, this maybe the source of wide inter-reader variabilities in this group. At the extremes are slides with no organisms scoring the same 4 points as slides with more than 30 lactobacilli and more than 30 small gram negative rods! The source of this confusion is the inverse relation that there is between lactobacillus and BV. Table 13 shows different score combinations that gives 4 points.

Table 13: Different score combinations leading to the same score in the Nugent intermediate group.

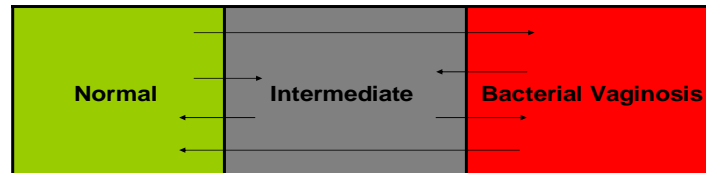
| Grade | Lactobacilli score | | Gram negative rods | points | Points |
|---------------------|---------------------------|---|---------------------------|---------------|---------------|
| 0 (no Organisms) | 4 | + | 0 | = | 4 |
| 1+ (<1 per field) | 3 | + | 1 | = | 4 |
| 2+ (2-5 per field) | 2 | + | 2 | = | 4 |
| 3+ (6-30 per field) | 1 | + | 3 | = | 4 |
| 4+ (>30 per field) | 0 | + | 4 | = | 4 |

To explain the variability the SLM was evaluated against Amsel on the intermediate group and the sensitivities, specificities and predictive values were 56%, 45%, 36% and 65% respectively (table 14) with a Kappa index of 0.01.

The intermediate group also includes the majority of cases that have only one or two positive Amsel criteria and therefore not enough to count as positives. Figure 2 below is an effort to try and describe the dynamic nature of BV status within a population regarding the intermediate group. We have participants who can move from normal directly to BV and vice versa (long arrows). For these the intermediate stage is only transient. We also have others that are moving back and forth between normal and intermediate and between full blown BV and intermediate (short arrows). When participants are intermediate it is difficult to tell which way the infection is heading, unless they are followed prospectively. We know that women may have recurrent BV but it is also important to establish whether there are chronic cases of BV and intermediate stages.

In most intermediate cases the densities of the organisms on the slide are so conspicuously varied such that it is almost impossible to get the same score from repeated readings of the same slide. This variation in the densities of the organisms is also present in the negative and the positive slides but the variation does not affect the score because the counts per field far exceed the >30 organism which is the magic number required to score 0 for lactobacillus or 4 for small gram variable rods.

Fig 2: Relationship between normal, intermediate and full blown BV



The Nugent intermediate group is difficult to diagnose and may be the source of inter and intra-reader variations. This was confirmed by the poor sensitivities, specificities and predictive values calculated using Amsel as the reference method (Table 14).

Table 14: Performance characteristics of the SLM on the intermediate category.

| | | Amsel Criteria | | |
|------------|--------------|-----------------------|-----------------|--------------|
| | | <u>Positive</u> | <u>Negative</u> | <u>Total</u> |
| SLM | Positive | 09 | 16 | 25 |
| | Negative | 07 | 13 | 20 |
| | Total | 16 | 29 | 45 |

SLM, Simple Lactobacillus Method.

These were 56%, 45%, 36% and 65% respectively with a Kappa index of 0.01.

The intermediate group may either represent a completely different type of abnormal vaginal flora namely aerobic vaginitis [11] which both the Amsel and Nugent criteria are not able to adequately address. The 30(11.5%) slides which are intermediate and the other 45 (19%) slides that were discrepant perhaps could have been reduced had we standardised the microscopic image area because different microscopes have magnifications which may differ from each other and from the original microscope

used by Nugent by as much as 300%[55]. This means that microscopes have an effect on the sensitivity, specificity and predictive values of Nugent criteria.

However the trend in BV diagnosis appears to be returning to the original diagnosis upon which Amsel evolved. Schmidt and Donders have explored various methods for the diagnosis of BV in wet mounts [39, 40, 48]. This seems to indicate that the solution to the diagnosis of bacterial vaginosis lies close to the definition of the condition, a shift in the micro ecology of the vagina. Amsel provides an accurate clinical description of bacterial vaginosis and can be used for diagnostic purposes but because of the subjective criteria that may affect its reliability we suggest that it must not be used as a reference method. Whilst we acknowledge that bacterial vaginosis is not a monoetiologic condition we would like to emphasize that certain organisms are key players in the aetiology of BV and these are lactobacilli and *G. vaginalis*. Lactobacilli are negatively associated with BV while *G. vaginalis* is positively associated. Therefore if one or both of these organisms are included in the diagnostic criteria it would certainly improve the diagnosis of bacterial vaginosis and hopefully improve reliability of the method.

Performance characteristics of individual criteria

Table 9 displays the individual performance of the four Amsel criteria and also that of lactobacilli and we observe that the sensitivity of lactobacilli comes second after pH, thus confirming why it should be included as one of the criterion for the diagnosis of bacterial vaginosis in wet preparations. Lactobacillus is an objective criterion and with minimum training it should provide results with minimum inter and intra-reader variability. However, the comparison in table 9 was made after removal of the intermediate group in order to make the Nugent result dichotomous. Even when it was compared with Amsel criteria (results not shown) the sensitivity of Lactobacilli was still second to pH, further confirming the need to include it as a criterion for the diagnosis of BV. Based on its individual performance we therefore replaced some of the Amsel criterion, one at a time, with lactobacilli and the two best combinations are shown in table 10, together with the conventional Amsel criteria in the first row. The combination with a higher bacterial vaginosis prevalence replaced discharge with lactobacillus while the other combination replaced the whiff test with lactobacilli. A participant was positive if at least any three positives out of four were as in the

original Amsel criteria. Both methods show a higher prevalence of bacterial vaginosis than Amsel criteria and this appears to indicate some level of misdiagnosis due to the subjective nature of some of the clinical criteria, [56] namely discharge and whiff.

Any three of whiff, clue cells, pH and lactobacillus for the diagnosis of BV was then compared against Amsel (any 3 of discharge, whiff, pH and clue cells) and Nugent criteria and the results are shown in table 15 below. With a near 100% sensitivity against Amsel this method is done at exactly the same cost with Amsel but has an advantage of having more objective criteria which means that it is more reliable. Another important objective criterion which must be considered for use is a group of small bacteria that adhere to each other, forming clumps and floating between epithelial cells [40]. These bacteria, pH, lactobacillus and clue cells would provide an all subjective criteria for the diagnosis of bacterial vaginosis.

Table 15: Combination of whiff, clue cells, pH and lactobacilli compared with Amsel and Nugent

| | Amsel n = 392 | Nugent n = 318 |
|---------------------------|-----------------------|-----------------------|
| Sensitivity | 0.99(95% CI = 98-100) | 0.76(95% CI = 71-81) |
| Specificity | 0.84(95% CI = 80-88) | 0.88(95% CI = 84-92) |
| Positive Predictive Value | 0.71 | 0.82 |
| Negative Predictive Value | 1.00 | 0.83 |
| <u>Kappa</u> | 0.71 | 0.64 |

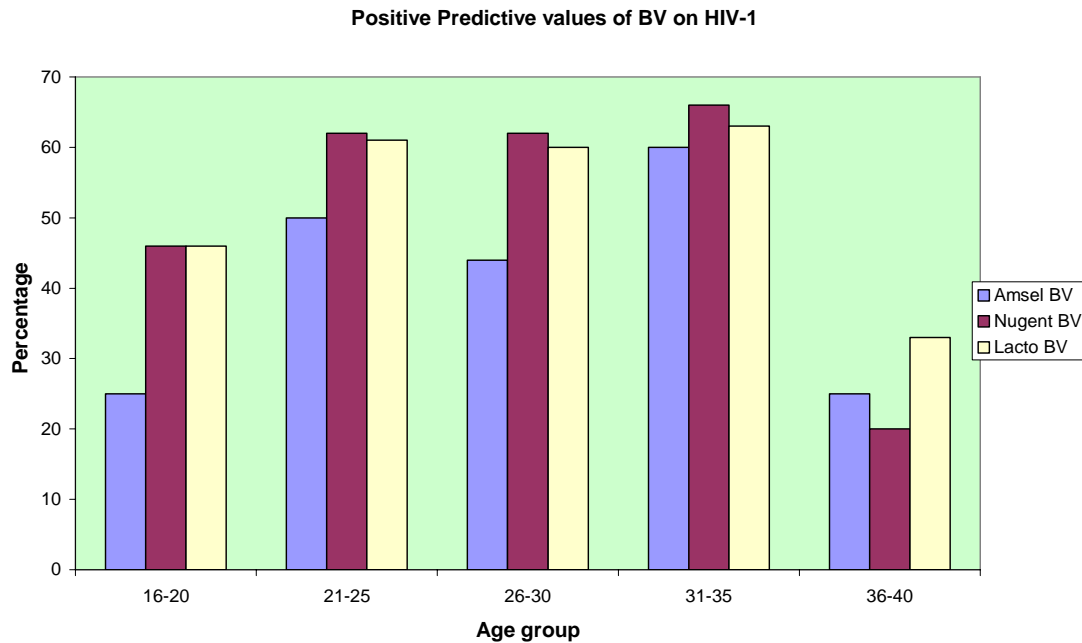
CI, Confidence Interval.

HIV, BV and lactobacilli

The simple lactobacilli method uses the presence or absence of lactobacilli as an indicator of abnormal vaginal flora and/or bacterial vaginosis. With a p-value far less than 0.0001 this study has demonstrated that the association between absence of lactobacilli and presence of HIV is highly significant. In 1999 the absence of lactobacilli in non pregnant asymptomatic women was associated with being positive for HIV (odds ratio 0.24)[13] while in this study the odds ratio on pregnant asymptomatic women is 2.14, an increase of almost ten times higher than the 1999 figure. A similar comparison between Amsel BV and HIV yields a p-value of 0.313, thus strangely indicating no association although several studies have reported an association [13, 14, 57]. Women without lactobacilli (odds ratio 2.14, p-value <0.0001) are more than twice likely to get HIV than women with bacterial vaginosis only (odds ratio 0.78, p-value 0.313). Absence of lactobacilli in the reproductive tract has a PPV of 56% for HIV infection. The relationship between HIV and BV among the age groups is shown in table 12 in the results section. If we look at the PPV of

Amsel, Nugent and the SLM for HIV we notice that Amsel has the least prediction for HIV across the age groups while Nugent and the SLM are both higher and comparable (fig 3). Both methods have over 60% PPV in the age groups between 21 and 35yrs. However, the SLM has an advantage over Nugent because it is much simpler to perform and therefore has a potential for a much wider application.

Fig 3. Comparison of Positive Predictive Values of Amsel, Nugent and the Simple Lactobacillus Method for HIV-1.



When confronted with a decimating disease like AIDS, whose treatment is currently not afforded by the majority of people in developing countries, it is much better and cheaper to identify individuals at risk and subject them to well structured preventive strategies which are intended to reduce transmission and increase knowledge. Although the disease burden is relatively small in the younger age groups it appears that they are the ones at a higher risk of acquiring infection.

Table 16: Cross tabulation table of the 88 mothers in the 16-20yr age group showing their lactobacilli BV and HIV status.

| | HIV Pos | HIV Neg | Total |
|--------------------------|---------|---------|-------|
| Lactobacilli BV Positive | 22 | 26 | 48 |
| Lactobacilli BV Negative | 10 | 30 | 40 |
| Total | 32 | 56 | 88 |

BV, Bacterial vaginosis.

There are 88 young mothers aged between 16 and 20yrs of which 32(36%) are HIV positive and 56 (64%) are HIV negative (Table 16). 22 (69%) of the HIV positive young mothers have no lactobacilli in the vagina while 26 (46%) of the HIV negative

young mothers also have no lactobacilli in the vagina. This means that these 26 young mothers are at a higher risk of acquiring HIV and must therefore be targeted for well structured preventive strategies. The aims of preventive strategies for these women must include restoration of the lactobacilli, couple counselling and condom use. It is also important to try and establish any behaviour, no matter how subtle, that may appear to be the cause of the reduction of lactobacilli. There are also 30 HIV negative women who still have lactobacilli in the vaginal ecosystem. These women have a relatively lower risk of acquiring HIV and the preventive strategies towards them must be different from those directed at those that have already lost their lactobacilli. Here the aim is to ensure that the vaginal ecosystem is maintained. When counselling the HIV negative women who still harbour lactobacilli it must be made quite clear that it is very possible to acquire HIV even with lactobacilli in the vaginal ecosystem. It is just the chance which is reduced but the possibility is there. This does not, however, imply that one has to lose lactobacilli first before acquiring HIV.

If we look at the individual years within the 16-20yr age group we notice that 2 of 6 (33%) are already infected at 16yrs while 10 of 24 (42%) are infected at 20yrs (table 17). The risk of getting infected with the virus is increasing with age. It is really not the age that matters but it is the increased frequency of having unprotected sex. It therefore appears that this age group is responsible for fuelling the spread of HIV. It has been documented that higher HIV seroconversion (transmission) is associated

Table 17: Distribution of HIV-1 infections within the 16-20 yr age group

| Age (yrs) | Number | <u>HIV-1 Status</u> | |
|-----------|--------|---------------------|----------|
| | | Positive | Negative |
| 16 | 6 | 2 (33) | 4 |
| 17 | 14 | 3 (27) | 11 |
| 18 | 19 | 9 (47) | 10 |
| 19 | 25 | 8 (32) | 17 |
| 20 | 24 | 10 (42) | 14 |
| Total | 88 | 32 (36) | 56 |

with a younger maternal age [57]. In a monogamous heterosexual relationship HIV transmission depends not only on the woman's behaviour but also on that of her partner(s) as well. We can also state that infection in the 16 year old mothers and perhaps in the whole age group is fairly recent because they have not been sexually active for a long time. They have relatively more sexually active years ahead of them

than the rest of the groups and must therefore be made prime targets for intervention programmes.

As these young mothers graduate into the next age group they bring with them their HIV status and affect both the prevalence and incidence in that group. Because they are sexually active they automatically become involved in the transmission dynamics of the virus. The positive will transmit and the negatives become the victims. This transmission is not only limited to their current age group but occurs across the entire sexually active age groups including those that are outside the reproductive ages. This helps to elaborate what is meant by fuelling the epidemic.

We would have wanted to show what is happening in the 31-40 year mothers but unfortunately the numbers are too few to make any meaningful conclusions. But we can speculate that very little transmission goes on in this age group but this is where the disease burden is quite evident. Those that are positive are probably graduating from the previous age groups and with death taking its toll they become fewer and fewer. The chances therefore of the negatives to seroconvert are relatively small unless something drastic happens to their behaviour and those of their partners.

This information may be very useful in revising existing HIV counselling scripts and other preventive strategies. It is important to bring to the attention of the women that although they are not HIV positive, but because of the absence of lactobacilli in the lower genital tract, if they continue to have unprotected sex, their chances of acquiring HIV are very high. Knowledge about the HIV-lactobacilli association may help reveal hidden and unknown risk factors that can be used to reduce HIV transmission. This knowledge should be used to complement the existing knowledge about the relationship between HIV and BV rather than replace it. If BV screening is made available women should be able to visit a clinic and ask for their lactobacillus status to be checked. There should be a deliberate effort by health care-givers to talk about vaginal health at primary health clinics. We need to deliberately target preventive strategies at women that are sexually active and are below the age of 20. In that age group we need to pay particular attention to women that do not have lactobacilli in the lower genital tract and those that have bacterial vaginosis. Eventually this may reduce transmission within this age group and indeed across all age groups.

The idea of targeted preventive strategies appears to provide a convenient entry point for vaginal health education to complement the existing reproductive health education whose main emphasis is to avoid teen age pregnancies, unwanted pregnancies and

subsequent abortions. Specific aids-related health education programs may be useful in arresting the spread of HIV by bringing to the attention of vulnerable women both silent and salient issues that puts them at risk. There may be traditional limitations to vaginal health but faced with the AIDS scourge we are forced to explore convenient ways of communicating the messages. Capitalising on the already existing relationship between mother and daughter can be a good starting point.

We observe that of the HIV positive women 59% do not have lactobacilli and 26% have bacterial vaginosis. Being pregnant is enough evidence that they are having unprotected sex. In a heterosexual relationship is the male partner at a higher risk of acquiring HIV by having sex with a woman who is HIV positive and is devoid of lactobacilli in the vagina or has bacterial vaginosis? Is there more viral shedding in the genital tract of HIV positive women devoid of lactobacilli?

3.2 Study Limitations

Our study participants were all enrolled in the third trimester of their pregnancy. This fact, coupled with the cross sectional nature of the study, in principle excludes the possibility of relating absence of lactobacilli to preterm effects on the BV study population. This study therefore failed to examine the effect of not having lactobacillus to early abortions, premature rupture of membranes and preterm births.

Although BV is the same in pregnant women as in non-pregnant women we are sceptical about generalising our results to non-pregnant women since pregnant women may behave hygienically different from non-pregnant women. The different behaviour may make one group more susceptible to BV acquisition than the other. For example, pregnant women may be involved in vaginal cleaning before visiting the clinic and this may affect the bacterial population. The study should therefore have included, as controls, non-pregnant women whose visit to the clinic was neither for obstetric nor gynaecologic reasons.

The study looked at only the presence or absence of lactobacilli. We did not count the number of lactobacilli per microscopic field and as a result we were not able to increase the sensitivity by including those with few lactobacilli per microscopic field as positives. We were also not able to establish cut-offs for the sensitivities.

The relationship between HIV and Lactobacillus could have been better demonstrated if the study was prospective and the participants randomised to one group receiving

extensive counselling to reduce HIV transmission and the other no counselling. HIV incidence would be evaluated after a given period in the two groups.

The technician received training rather late during the study and this could mean that the diagnosis was more accurate in the last set of slides than the initial ones. The same microscope was used for wet mounts but unfortunately three different microscopes were used for the Nugent Criteria and this may result in assigning different Nugent scores [55]. The delay in sending swabs to the laboratory may have lead to an under estimation of the whiff test as the volatile amines could have long evaporated in the heat by the time the swabs are delivered to the laboratory. The validity of clue cells and whiff test could have been enhanced tremendously if two technicians had read the wet mounts simultaneously.

3.3 Conclusion

The sensitivity and specificity of the lactobacillus method is comparable with both the Amsel and Nugent. In addition it has a potential to identify most women with abnormal vaginal flora. There is a strong association between absence of lactobacilli and presence of HIV-1. The Simple Lactobacillus method has shown a better positive predictive value for HIV-1 compared with both Amsel and Nugent. Further, it appeared that replacing discharge and whiff with lactobacillus one at a time is likely to improve the sensitivity of Amsel criteria to identify the majority of pregnant women with bacterial vaginosis.

3.4 Recommendations

The simple lactobacillus method should be further standardised by establishing cut-off values that can accurately distinguish bacterial vaginosis from other conditions that result from the loss of lactobacillus, such as aerobic vaginitis.

HIV negative women, especially those below the age of 30, without lactobacilli as part of the normal vaginal flora should be targeted for preventive strategies including extensive couple counselling to ensure reduced transmission. Furthermore, Ministries of Health, Education, Sports Youth and Culture and any other relevant ones should explore the best possible ways of introducing vaginal health education in the schools.

3.5 Future Study Topics

Atopobium vaginae, a strict anaerobic, metronidazole resistant, gram-positive coccobacillus has recently been found in the majority of women with bacterial vaginosis and in very few women without bacterial vaginosis. Its role in bacterial vaginosis is still not clear. The aim would be to find its prevalence in pregnant women and also its association with adverse pregnancy outcomes and infertility.

Aerobic vaginitis has been recently defined. How much overlap is there between bacterial vaginosis and aerobic vaginitis and what is the role of aerobic vaginitis in adverse pregnancy outcomes?

There is no data in Zimbabwe and the region on the association between BV and adverse pregnancy outcomes and vertical transmission of HIV from mother to child.

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Appendix 1

Gram reagents, staining procedure and reading instructions for Nugent criteria

Gram stain reagents

- Methanol (fixative)
- Crystal violet (primary stain)
- Iodine solution (mordant)
- Acetone (decolourizer)
- Safranin (counter stain)

Other supplies

- Light microscope with 100X objective
- Oil immersion
- Timer
- Filter paper
- Staining rack
- Fixing/staining trough with holder
- Differential counting machines

Procedure

Fixing

Arrange slides back to back on a slide holder and place in a trough containing methanol for 5 minutes. Remove slides and leave to dry freely in the air. If slides will not be stained immediately store them in a slide box labelled fixed. Include control slides during fixing, storage and staining.

Staining

1. Place between 5 and 10 slides on a staining rack and flood with crystal violet. Leave for 1 minute and wash with gentle running tap water. Shake excess water from the slide. (Do not stain more than 10 slides at a time.)
2. Flood the slides with iodine solution and leave for 1 minute. Rinse again with gentle running tap water. Shake excess water from the slide.

3. Hold slide with forceps and add acetone drop wise until all the blue colour has completely run out. Quickly rinse slide in gentle running tap water. Repeat for all the slides on the rack. Care must be taken not to over decolourise.
4. Flood slides with counter stain (safranin) and leave for 30 seconds. Rinse with gentle running tap water until all colour has run out. Shake off excess water.
5. Blot dry by pressing gently face down on blotting paper. Turn the slide face up and allow to air dry before viewing it under the microscope.

Reading slides according to Nugent's criteria

1. Scan the entire smear for adequacy of specimen. A smear must have several areas with cells and/or bacteria. An inadequate smear will have only mucus. Cells and bacteria will not be present.
2. Using 40x objective scan the entire slide to get a better sense of the organisms present. Swing the 100x objective into place and read five random fields from various parts of the slide. Look for big gram- positive rods, small gram-negative rods and curved gram-variable rods.
3. Use differential counting device to count the total number of each organism in the **five fields** and divide by five to obtain an average. Use the average to obtain a score of the individual bacteria as shown in the table below. Each slide will yield three sets of scores, a score for long gram-positive rods (lactobacillus), a score for short gram-negative rods (*G. vaginalis*) and finally a score for curved gram variable rods (*Mobiluncus*).

Add the individual scores to obtain the points for the slide. The points will determine the BV status of the client. Record result on appropriate record form.

Scoring guide used for the enumeration of bacteria for the Nugent's criteria.

| grade | Average number of bacteria per field | score | | |
|-------|--------------------------------------|----------|----------|----------|
| | | Gram +ve | Gram -ve | Gram +/- |
| 4+ | >30 | 0 | 4 | 2 |
| 3+ | 6-30 | 1 | 3 | |
| 2+ | 1-5 | 2 | 2 | 1 |
| 1+ | <1 | 3 | 1 | |
| 0 | 0 | 4 | 0 | 0 |

Appendix 2

Request to Participate.

The bacterial vaginosis study is an ancillary to the Mother To Child Transmission (MTCT) study in which you are already enrolled. Bacterial vaginosis is a shift in the vaginal flora and is characterised by a milky white malodorous discharge, elevated pH and presence of clue cells. Usually the odour is more noticeable after sexual intercourse. Strictly speaking BV is not an STI. The most common symptoms of bacterial vaginosis are milky white adherent discharge and a fishlike odour although most women that have bacterial vaginosis do not show any of these symptoms.

In pregnancy bacterial vaginosis is associated with amnionitis, chorioamnionitis, premature rupture of membranes, preterm births, low birth weight, intra-uterine deaths and stillbirths. It is also being increasingly associated with the acquisition of HIV-1 and other sexually transmitted infections.

There are many methods available for testing bacterial vaginosis but they are either too expensive or not suitable for the type of clinics we have in this country. In this study we want to see if we can come up with a simple method that can be used for the diagnosis of bacterial vaginosis in our clinics.

The specimens that you submitted in the main MTCT study are enough for us to do this study, in particular the vaginal swabs obtained during gynaecologic examinations. No extra specimens will be taken from you. No direct benefits may accrue to you individually except for the services that you are already getting in the main study and the transport reimbursement.

We are therefore asking for your permission to allow us to use your specimens for the bacterial vaginosis study.

You give us this permission of your own free will and you are free to stop us from using your specimens at any time. This will not in any way jeopardize the services that you often get from the clinic or the main study.

If you have any questions you can ask now or anytime as they arise.

.....tear
Consent form

Study ID

The study information has been read to me and I understand the aim of the study. I show my willingness for my specimens to be used by putting my signature or a thumb print on this form. This I do of my own free will.

Signature of participant

Date

Interviewer's signature

I testify that I have, to the best of my ability read and explained the information concerning this research study to the participant whose study ID appears above.

Interviewer's signature

Date